PATENT
Docket No.: **2354-380 (FF39178/06)**

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants	:	Pera *et al.*	Examiner: Michail A. Belyavskyi
Serial No.	:	10/574,885	
Filed	:	October 8, 2004	Art Unit: 1644
For	:	CELL SURFACE MARKER	

DECLARATION OF Martin Frederick Pera
UNDER 37 C.F.R. § 1.132

Commissioner for Patents
PO Box 1450
Alexandria, VA 22313-1450

Dear Sir:

I, Martin Frederick Pera, pursuant to 37 C.F.R. § 1.132, hereby declare:

1. I hold a PhD degree in Pharmacology from the George Washington University.
2. I am Professor of Cell and Neurobiology and Director of the Eli and Edythe Board Center for Regenerative Medicine and Stem Cell Research at the University of Southern California.
3. I am a co-inventor of the above-identified patent application.
4. I hereby submit experimental data which show that the antigen recognized by the GCTM-5 antibody referred to in the claims of US Patent Application 10/574,885 is biochemically distinct from the antigens recognized by the GCTM-1, -2, -3 and -4 antibodies disclosed in the prior art documents cited by the US Examiner against this application.

5. The Examiner's attention is referred to Figure 1, attached herewith, which shows an immunoblot of GCTM-5 antigen affinity purified from a CFPAC-1 pancreatic adenocarcinoma cell line condition media (CM) (see Figure 1, Panel C - Lane 1, untreated CM; lane 2 N-Glycanase (PNGase F) treated CM; lane 3, sialidase A treated CM; lane 4, O-Glycanase treated CM; lane 5, CM treated with all three enzymes). The data illustrated in Figure 1C demonstrate that sialidase treatment destroys GCTM-5 reactivity. By contrast, previous studies have shown that GCTM-2 antibodies bind to an antigen that is not sialidase sensitive. In fact, independent studies have shown clearly that sialidase treatment enhances the reactivity of GCTM-2 antibodies with its epitope (see Cooper *et al*, *J. Anat*, 200(3):p259, 2002) (attached hereto as Exhibit 1). Thus, the antigen identified and bound by the GCTM-5 antibody disclosed in US Patent Application 10/574,885 must be biochemically distinct from the antigen identified and bound by the GCTM-2 antibody of the prior art.

6. The Examiner's attention is also referred to Table 1, attached herewith, which illustrates the reactivity of the GCTM-5 antibody disclosed in US Patent Application 10/574,885 in normal and pathological human tissues. The data show that the GCTM-5 antibody has distinct patterns of tissue expression that are clearly different to those of the GCTM-2 antibodies of the prior art. For instance, GCTM-2 antibodies stain the surface of undifferentiated embryonal carcinoma stem cells and undifferentiated embryonic stem cells, whereas the GCTM-5 antibodies disclosed in US Patent Application 10/574,885 react only with a minority population of differentiating cells.

8. As further evidence for the distinct pattern of reactivity of GCTM-2 antibodies compared to the GCTM-5 antibodies disclosed in US Patent Application 10/574,885, the Examiner's attention is referred to Table 2 and Figure 2, attached herewith, which illustrate the reactivity of the GCTM-5 antibodies disclosed in US Patent Application 10/574,885 to fetal thymus and fetal pancreatic ductiles. This pattern of reactivity is clearly different to that of GCTM-2 antibodies, which do not

react with fetal thymus or pancreas (see Mason and Pera, *Eur. J. Cancer*, 28A(6-7):p1090, 1992) (attached hereto as Exhibit 2).

9. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

12 APR 10

Date

Martin Frederick Pera

FIGURE 1

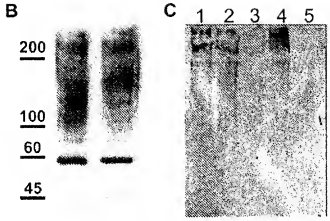

Figure 1. Immunoblot of GCTM-5 antigen affinity purified from CFPAC pancreatic adenocarcinoma cell line supernatant (A). (B) GCTM-5 immunoblot of CFPAC-1 conditioned media (CM). Lane 1, untreated CM; lane 2 N-Glycanase (PNGase F) treated CM; lane 3, sialidase A treated CM; lane 4, O-Glycanase treated CM; lane 5, CM treated with all three enzymes (B). Sialidase treatment destroys GCTM-5 reactivity

TABLE 1

NORMAL AND REGENERATING HUMAN TISSUE	n=	Extent of GCFM-6 REACTIVITY	COMMENTS - pattern of immunoreactivity (a= apical, c= cytoplasmic)
MESODERMAL/ECTODERMAL DERIVED			
Kidney	1/2	+/-	proximal tubule (c)
Bones and Normal Skin	2/2	-	adnexal structures (a)
Dermis	0/4	-	
Lymph Node	0/4	-	
Cerebral Cortex	0/2	-	
Cardiac Muscle	0/2	-	
Keratinising epithelia	0/4	-	
Vasculature	0/36	-	
Blood		-	
Smooth Muscle	0/5	-	
NEOPLASTIC HUMAN TISSUE			
BENIGN			
Gallbladder Adenoma	1/1	+++	proliferating ductal component (a)
Ampulla of Vater Adenoma	1/1	++	proliferating ductal component (a)
Benign Hepatic Adenoma	1/1	-	weakly reactive tumour cells (c)
MALIGNANT			
Hepatocellular Carcinoma (HCC)	2/3	++	tumour cells weakly reactive (c)
Colonic Adenocarcinomas	0/5	-	
Pancreatic Adenocarcinomas	4/4	+++	tumour cells strongly reactive (c,a)
Gastric Polyp	1/1	+	weak reactive mucin secreting cells
Endocervical Polyp	1/1	++	mucin secreting glands (a, c)

- = no staining + = weak, ++ = moderate, +++ = extensive

FIGURE 2:

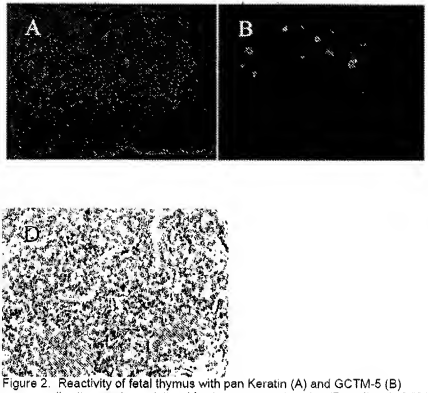

Figure 2. Reactivity of fetal thymus with pan Keratin (A) and GCTM-5 (B) antibodies, and reactivity of fetal pancreatic ductules (D, red) with GCTM-5.

Exhibit 1: Cooper et al., "Biochemical Properties of a Keratan Sulphate/Chondroitin Sulphate Proteoglycan Expressed in Primate Pluripotent Stem Cells," J. Anat. 200:259-265 (2002)

J. Anat. (2002) 200, pp259–265

REVIEW

Biochemical properties of a keratan sulphate/chondroitin sulphate proteoglycan expressed in primate pluripotent stem cells*

Susan Cooper,[1] William Bennett,[2] Jessica Andrade,[3] Benjamin E. Reubinoff,[3] James Thomson[4] and Martin F. Pera[3]

[1]University College London, London WC1, UK
[2]Department of Biology and Biochemistry, University of Bath, Bath BA2 7AY, UK
[3]Monash Institute of Reproduction and Development, Monash University, 246 Clayton Road, Clayton, Vic 3168, Australia
[4]Wisconsin Regional Primate Research Center, University of Wisconsin, 1220 Capitol Court, Madison, WI 53715, USA

Abstract

We previously identified a pericellular matrix keratan sulphate/chondroitin sulphate proteoglycan present on the surface of human embryonal carcinoma stem cells, cells whose differentiation mimics early development. Antibodies reactive with various epitopes on this molecule define a cluster of differentiation markers for primate pluripotent stem cells. We describe the purification of a form of this molecule which is secreted or shed into the culture medium. Biochemical analysis of the secreted form of this molecule shows that the monomeric form, whilst containing keratan sulphate, resembles mucins in its structure and its modification with O-linked carbohydrate. Immunofluorescence and immunoblotting data show that monkey and human pluripotent stem cells react with antibodies directed against epitopes on either carbohydrate side chains or the protein core of the molecule.

Key words chondroitin sulphate; embryo; embryonal carcinoma; embryonic stem cell; human; keratan sulphate; mucin; polylactosamine; proteoglycan.

Introduction

Primate pluripotent stem cell lines have now been developed from human embryonal carcinomas (ECs), monkey and human blastocysts, and human embryonic and fetal gonads (Thomson et al. 1995; Shamblott et al. 1998; Thomson et al. 1998; Pera et al. 2000; Reubinoff et al. 2000). The establishment of diploid human embryonic stem (ES) or embryonic germ cell lines has the potential to revolutionize biomedical research and to provide new opportunities for cell-based therapy. Although human ES cell cultures have been characterized using antibodies against cell surface antigens, the cultures are in fact heterogenous both in morphology and in terms of their surface antigen profile (unpublished observations). The biological significance of this heterogeneity is not yet understood, but it may be that differences in reactivity with various markers reflect differences in developmental potential amongst subpopulations within the cultures. A more precise definition of stem cell phenotype may assist in the development of more effective culture methodology for ES cells. The power of precise immunological analysis of cell differentiation lineage is clearly illustrated in the case of haematopoietic or lymphoid cells, where the structure of the stem cell populations at various levels of differentiation is well defined by expression of surface markers, most of which are characterized at the molecular level.

Previously, we identified a keratan sulphate proteoglycan present on the cell surface of human EC cells (Pera et al. 1988), tumour cells whose differentiation mimics early steps in mammalian development (Andrews, 1988). The proteoglycan was localized to the

Correspondence
Dr M. F. Pera, Monash Institute of Reproduction and Development, 246, Clayton Road, Clayton, Victoria 3168, Australia. Tel. +61 (0)39594 7318; fax +61 (0)39594 7311; e-mail: martin.pera@med.monash.edu.au

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pericellular matrix, and its expression was differentiation dependent: surface expression of the molecule on stem cells was lost following spontaneous or induced differentiation *in vitro* (Pera et al. 1988, 1989; Roach et al. 1994). This proteoglycan represents a surface antigen recognized by a cluster of monoclonal antibodies raised in different laboratories against human EC cells (Badcock et al. 1999). Using a monoclonal antibody reactive with the core protein, we found widespread expression in various epithelia of mid-trimester human fetal tissues (Mason & Pera, 1992), reminiscent of previous findings with monoclonal antibodies against keratan sulphate glycosaminoglycans (reviewed in Funderburgh, 2000).

In an earlier study, we reported the purification of the matrix-associated proteoglycan from human EC cells (Cooper et al. 1992). Much of the material so isolated was in an aggregated form. While keratan sulphate and chondroitin sulphate accounted for all of the glycosaminoglycan content of the pericellular matrix form, only chemical deglycosylation achieved complete removal of sugar residues, to reveal core protein bands of M, 55 and 48 kDa. We have observed previously that the proteoglycan could be detected in culture medium by immunoassay (Pera et al. 1988). Consequently, a new purification protocol was developed to study the secreted form of the molecule. The purified material was used as an immunogen in the production of a second monoclonal antibody, and the expression of the molecule on human ES cells and rhesus monkey ES cells was examined.

Materials and methods

Enzyme-linked immunosorbent assay (ELISA)

Enzyme-linked immunosorbent assay (ELISA) with the GCTM-2 antibody, reactive with an epitope on the proteoglycan core protein, and a monoclonal antibody against fibronectin (Sigma Chemical Co.) was carried out as described previously. The titre of proteoglycan immunoreactivity was estimated at various stages of the purification as described (Cooper et al. 1992).

Production of GCT 27 C-4 cell conditioned medium

The cell line GCT 27 C-4, a nullipotent clone of human EC cells (Pera et al. 1989), was subcultured at a 1 : 2 split ratio and grown overnight in a mixture of Minimal Essential Medium-Alpha and Ham's F12 medium (1 : 1 v/v) supplemented with 10% fetal calf serum, 1 mM glutamine and 1 μg mL^{-1} hydrocortisone. The cells were then washed twice with Iscove's Modified Dulbecco's medium supplemented with 35 μg mL^{-1} human transferrin, 5 μg mL^{-1} bovine serum albumin, 2.5 μg mL^{-1} human insulin, 1 mM glutamine and 1 μg mL^{-1} hydrocortisone, and were grown in this medium for 2–3 days. The conditioned medium was harvested, fresh medium was added and another harvest was carried out 2–3 days later.

Purification of immunoreactive proteoglycan from culture medium

Conditioned medium was filtered through Millex AP_{20} prefilters (Millipore Corporation) to remove cell debris. It was then passed through a 2–5 mL peanut lectin affinity column (Vector Laboratories) overnight at 40 mL h^{-1} at 4 °C. The column was washed with 10 volumes of 10 mM Tris/HCL, 150 mM NaCl, 0.1 mM $CaCl_2$, 0.01 mM $MnCl_2$ pH 7.4; subsequent procedures were carried out at room temperature.

Bound proteins were eluted with 0.6 M galactose in the wash buffer, collecting 0.5-mL fractions. Immunopositive fractions, as determined by ELISA, were pooled and diluted 1 : 1 with 50 mM Tris/HCL pH 6.8 (loading buffer). They were then loaded onto a 1-mL MonoQ anion exchange column on an FPLC apparatus (Pharmacia), and washed with 10 volumes of loading buffer. Proteins were eluted with a 0–1 M NaCl gradient. Strongly bound material was eluded with 2 M NaCl at the end of the run. The immunopositive material that eluted between 0.4 M and 0.6 M NaCl was pooled and concentrated using MicroSep concentrators (Flowgen) with a nominal M, cut-off of 30 kDa. This concentrated material was separated on a Superose 6 HR 10/10 column (Pharmacia), and 24 mL was equilibrated with 50 mM Tris/HCl, 0.5 M NaCl pH 6.8. Molecular mass markers used to calibrate the column were thyroglobulin, 669 kDa; ferritin, 440 kDa; and catalase, 232 kDa. The majority of immunoreactive material (eluting between 700 and 400 kDa) was pooled and diluted four-fold with 50 mM Tris/HCl pH 7.4 (final salt concentration 0.12 M NaCl), prior to affinity chromatography on a 1-mL heparin-Sepharose column (Pharmacia). The flow through was collected and subjected to further analysis. Bound human fibronectin was eluted from the heparin column with 2 M NaCl in 50 mM Tris/HCl, pH 7.4.

Purified proteoglycan was run on 7.5% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and then silver-stained and immunoblotted (Cooper et al. 1992).

Enzyme digestion with glycosidases

Purified proteoglycan was subjected to keratanase (keratan sulphate 1,4-β-D-galactohydrolase; EC 3.2.1.103; Pseudomonas sp., ICN Biomedicals Ltd) or chondroitinase (chondroitinase ABC lyase; EC 4.2.2.4; *Proteus vulgaris*, also from ICN) digestion followed by SDS/PAGE and immunoblotting as described previously (Cooper et al. 1992). Sequential digestion with sialidase (EC 3.2.1.18; *Arthrobacter ureafaciens*) and O-glycosidase (Endo-α-N-acetylgalactosaminidase; EC 3.2.1.97/3.2.1.110: *Streptococcus pneumoniae*), both from Oxford Glycosystems, was carried out as follows: 0.2 units of sialidase was reconstituted in 10 μL of reaction buffer (100 mM Na acetate pH 5.0). 5× reaction buffer was added to the proteoglycan followed by the sialidase and incubated at 37 °C for 2 h. O-glycosidase reaction buffer (5×, 100 mM Na citrate pH 6.0, 100 mg mL^{-1} bovine serum albumin, 0.02% sodium azide) was added to the mixture, followed by 6 mUnits of O-glycosidase, and incubated at 37 °C for 2 h. Finally, reducing SDS sample buffer was added and the samples were subjected to SDS/PAGE followed by immunoblotting as above.

Rotary shadowing electron microscopy

Purified intact proteoglycan samples were prepared for rotary shadowing as described previously (Cooper et al. 1992) and examined under a Philips EX301 electron microscope at a nominal magnification of ×45 000.

Production of a new monoclonal antibody

Except where noted, the standard immunological protocols described by (Harlow & Lane, 1996) were followed.

The secreted proteoglycan was purified as described above. The crude lectin-bound glycoprotein fraction from conditioned medium was used as the immunogen. Conditioned medium glycoproteins (6.5 mg) were mixed with the synthetic adjuvant Adju-Prime (Pierce Chemical Co.) in accordance with the manufacturer's instructions and injected intraperitoneally into female Balb/C mice approximately 6 weeks old. The mice were boosted twice with the same dose of immunogen alone administered intraperitoneally at biweekly intervals. Test bleeds, obtained after the second immunization, were screened by antibody capture ELISA against serial dilutions of the second major peak of proteoglycan immunoreactivity eluded from a Mono-Q Sepharose column as described above.

Spleen cells from the mouse whose serum displayed the highest titre of antibody against the semipurified proteoglycan were fused to NS-1 myeloma cells and hybrids selected using standard protocols. Supernatants from 472 wells containing approximately 1000 hybridoma clones were screened by ELISA against semipurified proteoglycan as described above; 48 positive wells were rescreened by ELISA and indirect immunofluorescence on methanol : acetone-fixed monolayers of GCT 27 C-4 cells (Pera et al. 1988). One hybridoma supernatant (TG 343) showed strong reactions in both of these assays.

Following subcloning by limiting dilution, the reactivity of TG 343 supernatant against semipurified proteoglycan and cultured cells was confirmed. TG 343 supernatant was next tested in an immunoblot assay against purified proteoglycan either in native form, or following treatment with keratanase or chondroitinase ABC as described above. The class and subclass of the antibody were determined using a commercially available kit (Amersham International).

Staining of rhesus monkey and human embryonic stem cells

Rhesus monkey embryonic stem cell line 278.5, and human embryonic stem cell lines HES-1 and HES-2 were cultured as described earlier (Thomson et al. 1995; Reubinoff et al. 2000). Human ES cell lines HES-3 and HES-4 were established from blastocysts as described (Reubinoff et al. 2000) and show expected properties of human ES cells including a diploid karyotype and formation of teratomas containing derivatives of all three germ layers in animal hosts; these cell lines were grown under the same conditions as HES-1 and HES-2. Immunostaining was performed as described previously. In some experiments the proportion of cells reactive with GCTM-2 or TRA-1-60 was estimated by harvesting cells using dispase and trypsin, staining in suspension and counting the number of positive cells under the fluorescence microscope. Immunoblotting

was carried out on extracts prepared from cell line HES-2 by extraction with 1.0% NP-40, 150 mM NaCl and 10 mM Tris-HCl, pH 7.5, followed by low-speed centrifugation to remove nuclei and debris. Proteins were separated on SDS-PAGE reducing gels, and transferred to membranes which were probed as described above.

Results

Purification of soluble proteoglycan

A combination of lectin affinity, anion exchange and gel filtration chromatography typically resulted in a purification of the proteoglycan from serum-free conditioned medium of approximately 5000-fold, with a yield of several per cent, as estimated from ELISA results (typical result for a 2.5-L preparation, Table 1). Losses were due in large part to heterogeneity in charge and size of the immunoreactive material bound to the lectin column. The preparation was subjected to SDS/PAGE followed by silver staining or immunoblotting (Figs 1 and 2 lane A, Fig. 3 lane A) and was shown to correspond to a single broad band of M_r approximately 200 kDa with very little immunoreactive material in the stacking gel. Fibronectin present in the immunoreactive peak from gel filtration was removed by heparin affinity chromatography with a loss of proteoglycan of about 15%, the amount that remained bound to the column in the presence of 1 M NaCl (not shown).

Deglycosylation of soluble proteoglycan

Both chondroitinase (data not shown) and keratanase (Fig. 2 lane B) partially deglycosylated and enhanced the immunoreactivity of the proteoglycan, evidenced by an increase in electrophoretic mobility on SDS-PAGE and a stronger reaction in immunoblotting. Results with chondroitinase were more variable than with keratanase. A combination of sialidase followed by O-glycosidase similarly enhanced the immunoreactivity of the purified material and increased its electrophoretic

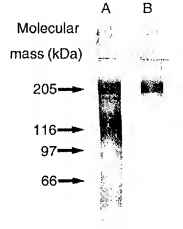

Fig. 1 7/5% SDS-PAGE of the MonoQ anion exchange pooled fractions (A) and the Superose 7 pool (B), followed by silver staining.

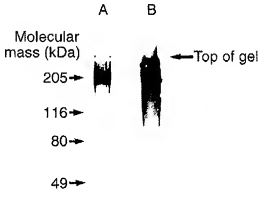

Fig. 2 Immunoblotting of purified proteoglycan before (A) or after (B) keratanase digestion.

Table 1 Estimate of purification of the proteoglycan from conditioned medium of GCT 27C-4 cells

Step	Volume (mL)	Protein* ($\mu g\ mL^{-1}$)	Relative titre†	Yield (% of initial)	Purification
Medium	2500	50	1.0	100	1
Lectin affinity	12.5	10	64	32	320
Anion exchange	0.5	30	256	5	425
Gel filtration	0.5	1.5	128	2.6	4270

*Based on OD 280 nm. †Estimated by ELISA.

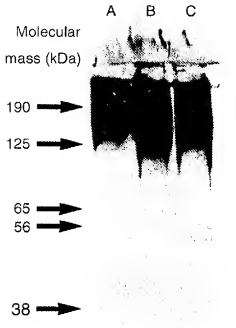

Fig. 3 Immunoblotting of purified proteoglycan before treatment (A), after sialidase treatment (B) or after sialidase followed by O-glycosidase treatment (C).

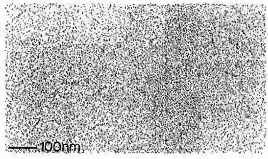

Fig. 4 Electron micrograph of rotary shadowed proteoglycan preparation.

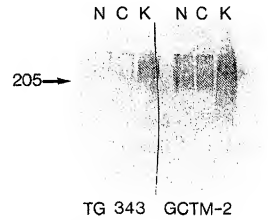

Fig. 5 Immunoblot analysis of purified proteoglycan with monoclonal antibodies TG343 and GCTM-2. Purified proteoglycan in its native form (N), or following treatment with chondroitinase ABC (C) or keratanase (K), was electrophoresed on a 7.5% SDS-PAGE gel under reducing conditions, transferred to immobilon membrane, and probed with antibody TG343 (left) or GCTM-2 (right). The position of the 205-kDa molecular weight marker is indicated by the arrow.

mobility, and converted a proportion of the purified proteoglycan to two immunoreactive bands of M, 55 and 48 kDa (Fig. 3 lane B). Two bands of similar size were obtained previously following chemical deglycosylation of the cell surface proteoglycan (Cooper et al. 1992).

Electron microscopy of soluble proteoglycan

The purified proteoglycan molecules that ran on Superose 6 gel filtration at ~700 kDa appeared fibrillar with a length of about 80 nm and thickness of 3.5 nm (Fig. 4). This is in contrast to the proteoglycan purified from the cell surface (Cooper et al. 1992) the majority of which formed long filamentous aggregates several micrometres in length.

Production of a new monoclonal antibody

The availability of the purified proteoglycan enabled us to produce a new mouse monoclonal antibody for further immunocytochemical studies, Clone TG343. Both antibodies reacted with the native proteoglycan in this assay (Fig. 5). Pretreatment of the proteoglycan with chondroitinase ABC, which sometimes produces a modest degree of enhancement of immunoreactivity with GCTM-2, had no effect on its reaction with either antibody in this experiment, indicating that the new antibody was probably not directed against chondroitin sulphate chains. Pretreatment with keratanase enhanced the immunoreactivity of the proteoglycan with both antibodies, and resulted in visualization of antigen with greater electrophoretic mobility, in some

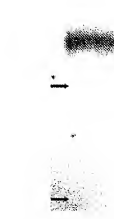

Fig. 6 Immunoblot of detergent-soluble extract from human ES cell line HES-2. The arrow indicates the position of 173-kDa marker protein.

blots, enzymatic treatment degraded material reactive with either antibody down to the size of the protein core. However, ELISAs in which GCTM-2 and TG 343 were allowed to complete for binding to immobilized semipurified proteoglycan showed that TG 343 did not interfere with GCTM-2 binding (not shown). Thus it is unlikely that the epitopes identified by the two antibodies are identical.

Reactivity of primate pluripotential cell lines with antibodies to the proteoglycan

Feeder-dependent human embryonal carcinoma cell line GCT-27X-1, rhesus monkey ES cell line 278.5 and human ES cell lines HES1, 2, 3 and 4 were examined by indirect immunofluorescence microscopy for reactivity with antibodies against several monoclonal antibodies reactive with the proteoglycan. The majority of cells in cultures fixed 5–7 days after subculture under conditions optimal for stem cell renewal were stained with all antibodies and the staining had a similar appearance in all cell lines: a granular staining outlining the cell body, often with a dark area in the nucleus, and with some deposition onto the culture surface. In the case of the human ES cell lines HES-1 and HES-2 the proportion of cells positive with TRA1-60 was consistently higher (70% vs. 50%) than that stained with GCTM-2. The proportion of cells staining with TG 343 was similar to that stained with GCTM-2. Immunoblotting of detergent-soluble extract from cell line HES-2 revealed that GCTM-2 reacted with a broad band of around 200 kDa (Fig. 6).

Discussion

Biochemical properties of the secreted proteoglycan

The proteoglycan studied here exits in several forms, depending on the source from which it is isolated. The properties of the soluble form of the molecule differ from those of the previously described pericellular matrix form in several respects. In preparations from conditioned medium, very little immunoreactive material remained in the stacking gel during SDS-PAGE, the majority migrating as a broad band of approximately 200 kDa, in contrast to the pericellular matrix form. The soluble molecules are less highly charged than those on the cell surface, most eluting at a lower ionic strength from anion exchange columns. We noted previously that if subconfluent monolayers were extracted with 1 M guanidinium hydrochloride, some of the immunoreactivity eluted at lower ionic strength during ion exchange chromatography, and was smaller than the bulk of the insoluble material. It is possible that upon reaching confluence cells begin to assemble the proteoglycan monomers into a large pericellular matrix complex.

Rotary shadowing confirms the difference between the two forms. The majority of the proteoglycan purified from the cell surface formed long filamentous aggregates several micrometres in length although a few putative monomers approximately 80 nm long and 3.5 nm wide were observed. Most of the material in the soluble form was monomeric. Formation of large aggregates is also observed for some secreted mucins (Strous & Dekker, 1992), which the proteoglycan resembles in appearance, but mucin aggregates are generally formed by intermolecular disulphide bonds. The aggregation observed in this proteoglycan is not due to disulphide linkages, or to hydrophobic interactions, since neither reducing conditions nor detergents can disaggregate the molecules.

Both chondroitinase (data not shown) and keratanase (Fig. 4B) partially deglycosylated and enhanced the immunoreactivity of the proteoglycan. A combination of sialidase followed by O-glycosidase similarly enhanced the immunoreactivity of the purified material and increased its electrophoretic mobility, and converted a proportion of the purified proteoglycan to two immunoreactive bands identical in size to those obtained by chemical deglycosylation of the cell surface proteoglycan (Cooper et al. 1992).

This development of this purification methodology, and the production of a new monoclonal against the

core protein should facilitate molecular cloning of the protein core and analysis of its function in pluripotent stem cells.

Expression in primate pluripotent stem cells

These studies extend and confirm previous results which demonstrate that this proteoglycan is a major surface antigen of primate pluripotent stem cells. Reactivity with antibodies recognizing both the core protein and carbohydrate epitopes, plus the immunoblotting data, strongly indicate that normal primate pluripotent cells express a molecular form of this molecule similar to that found on embryonal carcinoma cells. The proportion of cells stained with antibody TRA1-60 was somewhat higher than that seen with antibody GCTM-2; neither stained all cells in ES cultures maintained under optimal conditions. It is possible that the differences in reactivity with the two antibodies reflect some type of epitope masking in different cell types. The biological significance of this antigen heterogeneity remains to be determined, but the antibodies described herein will help determine the developmental potential of the subpopulations of cells within the cultures. Further study of this proteoglycan may also elucidate its function on the surface of pluripotent cells.

Acknowledgments

This work was begun with support from the Cancer Research Campaign, UK, and has been further supported by the National Health and Medical Research Council.

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Exhibit 2: Mason et al., "Immunohistochemical and Biochemical Characterisation of the Expression of a Human Embryonal Carcinoma Cell Proteoglycan Antigen in Human Germ Cell Tumours and Other Tissues," Eur. J. Cancer 28A(6-7):1090 (1992)

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Immunohistochemical and Biochemical Characterisation of the Expression of a Human Embryonal Carcinoma Cell Proteoglycan Antigen in Human Germ Cell Tumours and other Tissues

Malcolm D. Mason and Martin F. Pera

In the embryonal carcinoma (EC) cell line GCT 27, monoclonal antibody GCTM-2 recognises an epitope on a 200 kD pericellular matrix keratan sulphate proteoglycan. Immunohistochemical analyses demonstrated staining of tissue sections from 21 out of 22 human non-seminomatous germ cell tumours, and from 22 out of 28 sections of seminomas. In normal human fetal tissues gut epithelium and muscle stained strongly, and certain other epithelia stained moderately. In adult tissues, the distribution of the epitope was similar, but staining intensity was weaker. Neoplastic tissues showed reactivity with embryonal rhabdomyosarcoma and colorectal carcinoma, but no other non-germ cell tumours. Immunofluorescence microscopy showed that GCTM-2 also stained cell lines from human colorectal carcinoma, embryonal rhabdomyosarcoma and choriocarcinoma. In contrast to EC cells the epitope in these other cell types required permeabilisation of the cells to be visualised, and the protein bands in immunoblots lacked extensive modification with keratan sulphate and were smaller. Thus, GCTM-2 reacts with an epitope which has a previously unrecognised tissue distribution; its expression as a pericellular matrix proteoglycan is predominantly a characteristic of human EC cells.
Eur J Cancer, Vol. 28A, No. 6/7, pp. 1090–1098, 1992.

INTRODUCTION

THE SERUM markers α-fetoprotein and human chorionic gonadotropin are useful in monitoring patients with testicular non-seminomatous germ cell tumours (NSGCT) [1]. These secreted polypeptides are products of differentiated yolk sac cells and trophoblastic lineage respectively, which appear in a proportion of testicular teratomas [2]. Despite the utility of these markers, it would be desirable for cell biological studies and for certain clinical applications to define markers expressed on embryonal carcinoma stem cells themselves, rather than on their differentiated derivatives. A preliminary study has suggested that the GCTM-2 antigen merits investigation as one such clinical marker [3].

A number of cell surface antigens defined by monoclonal antibodies have been described in association with embryonal carcinoma. Many of these monoclonals react with carbohydrate antigens and are of the IgM class [4]. Monoclonal antibody GCTM-2 was shown to recognise a pericellular matrix EC proteoglycan which was susceptible to degradation by keratanase but not other glycohydrolases or lyases [5]. More recent studies on the purified GCTM-2 antigen confirmed that it is a keratan sulphate proteoglycan. The further evidence supporting this conclusion included aminoacid and sugar analyses of the antigen; the reactivity of the antigen with Alcian blue dye; high affinity of the antigen for anion exchange resins; metabolic labelling of

Correspondence to M. D. Mason.
M.D. Mason is at the Velindre Hospital, Whitchurch, Cardiff, South Glamorgan CF4 7XL, U.K. and M.F. Pera is at the Cancer Research Campaign, Department of Zoology, University of Oxford, South Parks Road, Oxford OX1 3PS, U.K.
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the antigen with inorganic sulphate and release of this labelled sulphate following keratanase treatment and the appearance of aggregates of purified antigen in electron microscopy (S. Cooper *et al.* Dept of Zoology, Oxford University. *Biol J* in press). The epitope recognised by the GCTM-2 antibody probably lies on the proteoglycan core protein, since the antigen–antibody interaction is enhanced by keratanase treatment, but is not altered by *N*-glycosidase digestion, or mild alkaline hydrolysis, which would be expected to release *N*-linked or *O*-linked carbohydrate, respectively; chondroitinase ABC and heparitan sulphate lyase also had no effect on the antigen–antibody interaction [5, and Pera, unpublished observations].

Proteoglycans are a diverse group of molecules with a range of functions, and are most often found in the extracellular matrix [6]. They are composed of one or more glycosaminoglycan chains covalently bound to a core protein, frequently with *N*-linked or *O*-linked oligosaccharides [7]. Given recent evidence that proteoglycans are important in the regulation of growth and differentiation [8], it is noteworthy that the GCTM-2 antigen disappears from the cell surface during spontaneous differentiation *in vitro* of the embryonal carcinoma cell line GCT 27X-1, a clonal derivative of GCT 27 [9].

In these studies we sought to further characterise the tissue distribution and biochemical characteristics of the antigen recognised by GCTM-2.

MATERIALS AND METHODS

Cell culture

GCT 27 cells were maintained in culture under the conditions described previously [10]. On reaching confluence the cells were harvested with 0.05% trypsin and 0.02% EDTA and replated at a 1:10 split ratio. The HX 18 cell line was established from a human colorectal carcinoma [11] and was grown under the same conditions as described for GCT 27, except that confluent monolayers were subcultured at a split ratio of 1:4. The cell line HX 170 was established from xenografts in nude mice of a human embryonal rhabdomyosarcoma and was grown as described [12]. The human choriocarcinoma cell line BeWo [13] was obtained from the European Collection of Animal Cell Cultures (Porton Down, Salisbury). BeWo cells were grown in Ham's F12 medium supplemented with 20% fetal calf serum, glutamine at a concentration of 2 mmol/l, and hydrocortisone at 1 mg/ml. The cells were subcultured when subconfluent at a split ratio of 1:6. Mouse hybridoma GCTM-2 [5] was grown in suspension in RPMI 1640 medium supplemented with 10% fetal calf serum (Myo-clone, Gibco). Cells were harvested twice weekly, and replated at a split ratio of 1:10. The cell supernatant was harvested, supplemented with 1 mmol/l phenylmethyl sulphonyl fluoride (PMSF) and used in this form.

Tissue samples

Specimens taken at orchidectomy for removal of a testicular tumour were fixed in 100% ethanol, and embedded in paraffin blocks. Additionally, blocks from formalin-fixed testicular tumours kept in histopathology laboratory archives were obtained from 15 specimens, and sections were cut from these blocks for immunohistochemical study. Germ cell tumours were classified histologically according to the WHO system [14]. Midtrimester fetal tissue was obtained from the tissue bank at the Royal Marsden Hospital (approval was obtained from the Royal Marsden Ethics Committee for the use of this material in this study). The specimens were dissected, fixed in 100% ethanol, and embedded in paraffin blocks before sectioning. Normal adult tissue was obtained at surgery, when its resection formed part of the procedure. It was fixed in 100% ethanol, and embedded as described above. Additionally, some normal adult tissue was obtained from formalin-fixed autopsy specimens. Non-germ cell tumours were obtained from formalin-fixed material held in the archives of the histopathology department of the Royal Marsden Hospital.

Immunohistochemistry

5 μm sections were cut from the blocks onto glass slides, dewaxed in three changes of xylene, and then rehydrated by passing them through 100, 70 and 40% (vol:vol) ethanol in water, and then through a final rinse in distilled water. Rehydrated sections from formalin-fixed material were treated with 0.1% Bacto Trypsin (Difco Laboratories) at 37°C for 1 h prior to staining, as preliminary studies had shown this to enhance staining to the levels seen with ethanol-fixed material. Control sections were treated with normal mouse serum diluted 1:400 in phosphate-buffered saline (PBS) plus 1% bovine serum albumin (BSA) as a source of control mouse immunoglobulin of the same class as GCTM-2 (IgM). GCTM-2 was applied to the test section as neat hybridoma supernatant. The slides were incubated at room temperature for 1 h (ethanol-fixed material) or for 1.5 h (formalin-fixed material). The slides were rinsed in PBS/BSA between this and each subsequent step. Next, rabbit anti-mouse immunoglobulin conjugated to biotin (Amersham) was applied to the sections at a 1:100 dilution in PBS/BSA, and incubated for 30 min at room temperature. Streptavadin conjugated to horseradish peroxidase (Amersham) was applied at a concentration of 1:300 vol/vol in PBS/BSA and incubated for 30 min at room temperature. Finally, 3,3-diaminobenzidine (Sigma), 0.5 mg/ml in PBS, plus hydrogen peroxide to a final concentration of 0.02% vol/vol, was added as substrate, and incubated for 3 min. The sections were counterstained with haematoxylin, dehydrated in two changes of 100% ethanol followed by 100% xylene, and mounted in D.P.X. (BDH). Those control sections in which extensive staining was observed (usually associated with massive necrosis) were deemed to be unassessable and were excluded from the analysis. Staining with GCTM-2 was only scored as 'positive' where no such staining was observed in corresponding control sections.

Indirect immunofluorescence

Cultured cells were grown on sterile multiwell slides (Flow) under the conditions described above. When grown to a suitable density, the cells were fixed in a 1:1 (vol:vol) solution of methanol/acetone over solid CO_2, and were stored at −20°C. Slides were stained and mounted as described [5]. For immunofluorescent staining of live cells, 10^6 cells were harvested, and resuspended in fresh medium. GCTM-2 antibody was added to the cell suspension to a dilution of 1:3. The cells were incubated at 4°C for 30 min, rinsed in PBS after centrifugation at 500 g, and resuspended in ice cold PBS. After a second wash, they were resuspended in diluted anti-mouse immunoglobulin/fluorescein isothiocyanate conjugate as described [5], and incubated at 4°C for 30 min. They were then mounted and viewed as described above.

Immunoblotting

Monolayer cultures of GCT 27, HX 18, HX 170 and BeWo were treated with a small volume of 50 mM Tris-HCl buffer, pH 7.4, with or without keratanase (keratan sulphate 1.4-β-D-galactanohydrolase from *Pseudomonas* sp. IFO-13309, ICN

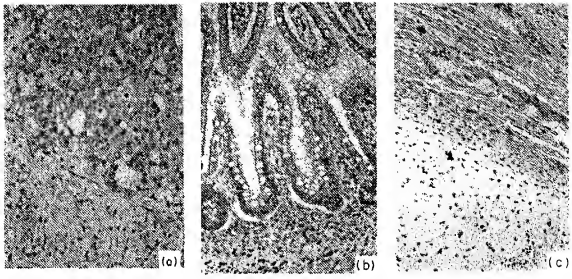

Fig. 1. (a) Human testicular teratocarinoma stained with GCTM-2. Embryonal carcinoma is stained but not surrounding mesenchymal tissue. (b) Second-trimester human fetal small intestine showing intense staining of mucosal epithelium by GCTM-2. (c) Second-trimester human fetal laryax stained with GCTM-2. Skeletal muscle fibres are stained but not adjacent cartilage.

Biomedicals) to a final concentration of 2.5 units/ml, and incubated for 1 h at 37°C. The preparation was then harvested into a small volume of sodium-dodecyl sulphate (SDS) containing sample buffer plus PMSF, sonicated, boiled for 5 min, and then run on 10% polyacrylamide gels [15]. Prestained marker proteins applied to the gel were α-macroglobulin, β-galactosidase, fructose-6-phosphate kinase, pyruvate kinase, fumarase, lactate dehydrogenase and triosephosphate isomerase (Sigma). Following separation, the proteins were transferred to a nitrocellulose membrane using flat graphite electrodes (Pharmacia), and the blot was processed and stained with GCTM-2, biotinylated anti-mouse immunoglobulin, and streptavadin peroxidase [5].

RESULTS

Immunohistochemistry on germ cell tumours

12 out of 13 NSGCT stained with GCTM-2; the single specimen which did not stain consisted of mature tissues from a specimen of differentiated teratoma. Furthermore, staining was also seen in 17 out of 23 seminomas, and in all three sections from tumours consisting of combined seminoma/NSGCT. Following treatment of formalin-fixed sections with PBS/trypsin, each of nine NSGCT, five seminomas, and one intratubular germ cell tumour (in which the subtype could not be determined) stained with GCTM-2. Figure 1a shows a section of teratocarcinoma stained with GCTM-2 in which there is staining of areas of EC but not of the surrounding primitive mesenchyme. Within the mesenchyme, staining was observed in a few isolated cells of uncertain nature. The staining pattern of EC cells was cytoplasmic as well as surface; this is in keeping with the known natural history of some extracellular matrix proteoglycans, which are assembled from core proteins in the cytoplasm before being packaged and exported to the cell surface [6, 7]. Staining was also observed in areas of carcinoma *in situ* where these were present.

Immunohistochemistry on mid-trimester fetal tissue

Table 1 summarises the results of immunohistochemical staining of ethanol-fixed specimens of second-trimester fetal tissue. The strongest staining was seen in gut epithelium, and in all types of muscle (skeletal, smooth and cardiac). Several other epithelia also stained more weakly with GCTM-2. Within the epithelium of the small bowel, it was noted that the strongest staining was generally seen at the apex of the villi, while many of the crypts stained relatively less strongly. No staining was observed in mesenchyme, cartilage, brain, lymph node, dermis, renal glomeruli or testis. Figure 1b shows a section of fetal small intestine with strong staining of the mucosal epithelium. Figure 1c shows a section of fetal laryax in which staining of skeletal muscle is observed but not of the adjacent cartilage.

Immunohistochemistry on adult normal tissues

Table 2 lists reactions of adult normal tissues with GCTM-2. Wherever possible these were obtained during surgery and fixed immediately in 100% ethanol. Some tissues (brain, breast, liver, testis, pancreas and lymph node) were obtained from formalin-fixed material obtained at surgery or post-mortem. The pattern of reactivity resembled that of fetal tissues, but the level of staining was generally much less intense.

Immunohistochemistry on non-germ cell tumours

The results of immunohistochemistry on sections from formalin-fixed non-germ cell tumours is shown in Table 3. The only tumours in which staining was seen were colonic adenocarcinoma, and embryonal rhabdomyosarcoma. No staining was seen in a variety of other tumours, including several which might be considered in the diagnosis of a malignancy of uncertain origin in a child or young adult.

Immunofluorescence studies on cultured cells

Fixed cell preparations from GCT 27, HX 18, HX 170 and BeWo all stained with GCTM-2 by indirect immunofluorescence (Fig. 2). In contrast, when suspensions of live cells were stained, though 100% of GCT 27 cells examined were reactive, only 30% of HX 18 cells, and none of the HX 170 or BeWo cells reacted with GCTM-2 (Fig. 3). This suggests that the epitope is on the

Table 1. Immunohistochemistry on second trimester fetal tissue

	No. positive/ no. tested
Strongly positive	
Gut epithelium	3/3
Muscle	10/10
Moderate	
Trophoblast	4/4
Liver	4/4
Kidney (epithelium of distal tubules and collecting ducts)	3/3
Weak	
Lung epithelium	2/2
Bladder epithelium	2/2
Oesophageal epithelium	2/2
Tracheal epithelium	2/2
Thyroid	2/2
Negative	
Cornea	0/2
Brain	0/2
Cartilage	0/4
Skin	0/3
Mesenchyme	0/2
Testis	0/3
Spleen	0/2
Pancreas	0/2
Thymus	0/2
Lymph node	0/2
Renal glomeruli	0/3
Adrenal	0/2

Table 2. Immunohistochemistry on adult normal tissues

	No. positive/ no. tested
Strongly positive	None
Weakly positive	
Colonic mucosa	2/2
Skeletal muscle	3/3
Smooth muscle	3/3
Renal tubules	1/2
Liver	2/2
Pancreas	2/2
Breast epithelium	2/3
Adrenal Cortex	2/2
Negative	
Skin	0/3
Tongue epithelium	0/2
Bladder epithelium	0/2
Mesenchyme	0/2
Testis	0/2
Brain	0/2
Lymph node	0/2
Renal glomeruli	0/2

Table 3. Immunohistochemistry on non-germ cell tumours

	No. positive no. tested
Strongly positive	
None	
Moderately positive	
Colon carcinoma	1/4
Embryonal rhabdomyosarcoma	2/3
Negative	
Adenocarcinoma breast	0/3
kidney	0/2
submandibular gland	0/1
Transitional cell carcinoma—bladder	0/3
Squamous carcinoma—head and neck	0/2
Ewing's sarcoma	0/2
Neuroblastoma	0/2
Hodgkins disease	0/2
Non-Hodgkin lymphoma	0/2
Wilms' tumour	0/2
Malignant melanoma	0/2

surface of all GCT 27 cells, 30% of HX 18 cells, and is inaccessible in live HX 170 and BeWo cells.

Immunoblotting of whole cell lysates

Immunoblotting on whole cell lysates from GCT 27 yielded a characteristic band at 200 kD, which was degraded on pretreatment of the cell lysate with keratanase (Fig. 4a), down to a size limit of 55 kD, as previously reported [5]. In this series of experiments immunoblotting also revealed staining higher than the 200 kD position, a feature that has been noted with immunoblots on purified GCTM-2 proteoglycan preparations, and which appears to be due to the tendency for the proteoglycan to self-aggregate (S. Cooper, *et al.*, Dept Zoology, Oxford University. *Biol J* in press). Immunoblotting on whole cell lysates from HX 18, HX 170, and BeWo yielded single bands at approximately 55–70 kD, and in contrast to GCT 27, pretreatment of the cell lysates with keratanase did not modify the position of the band or the intensity of the staining (Fig. 4b,c,d). However, preparations from HX 18 also yielded faint immunoreactivity at approximately 200 kD, which was more apparent in the keratanase-treated lysate (Fig. 4c).

DISCUSSION

Screening of a panel of cell lines with monoclonal antibody GCTM-2 showed that the epitope was expressed in embryonal carcinoma, visceral yolk sac carcinoma, and a poorly differentiated colorectal carcinoma [5]. Previously published biochemical and electron microscopic studies have indicated that the GCTM-2 antigen lies on a pericellular matrix keratan sulphate proteoglycan in human EC cells [5], and more recent work has confirmed the proteoglycan nature of the antigen and its keratan sulphate glycosaminoglycan content (S. Cooper *et al.*, Dept Zoology, Oxford University. *Biol J* in press).

In this study we have shown that in histological sections of testicular tumours GCTM-2 strongly stained embryonal carcinoma, and, in the differentiated areas of one teratocarcinoma, primitive muscle and columnar epithelium, in keeping with the distribution of staining seen in fetal tissues. Additionally,

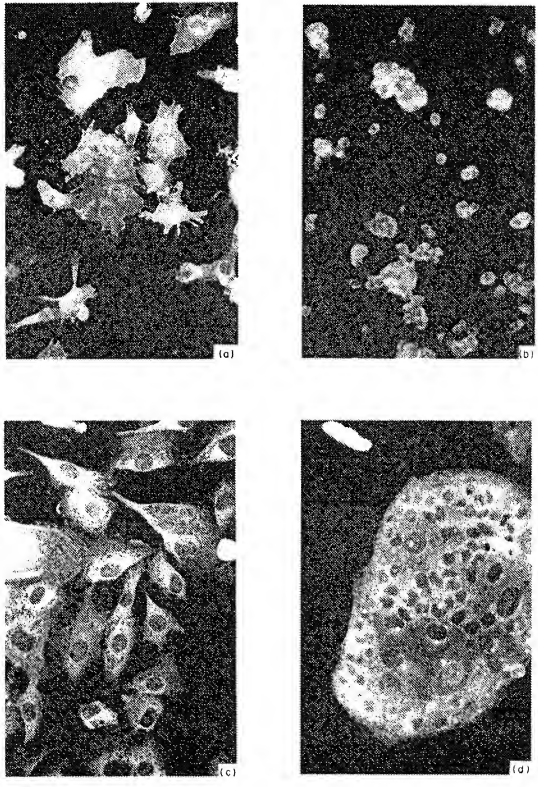

Fig. 2. Immunofluorescence on fixed cells with monoclonal antibody GCTM-2: (a) GCT 27; (b) HX 18; (c) HX 170; (d) BeWo.

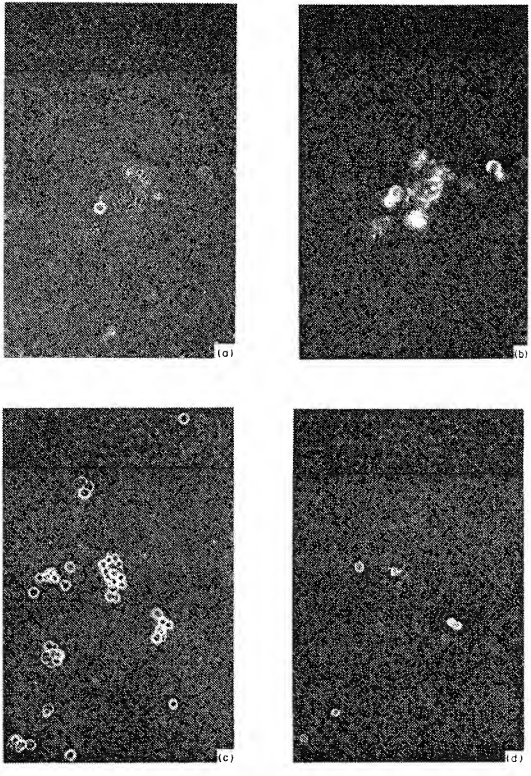

Fig. 3. Continued overleaf.

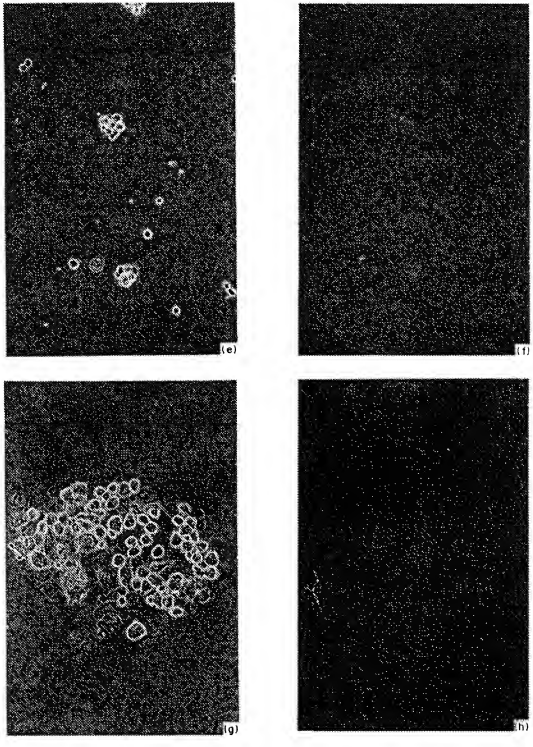

Fig. 3. Immunofluorescence on live cells with GCTM-2: (a) GCT 27 under phase-contrast; (b) same field as (a) under fluorescence; (c) HX 18 cells under phase-contrast; (d) HX 18 cells under fluorescence showing staining of subpopulation of cells in same field as (c); (e) HX 170 cells under phase-contrast; (f) HX 170 cells under fluorescence showing no staining; (g) BeWo cells under phase-contrast; (h) BeWo cells under fluorescence showing no staining of same field as (g).

GCTM-2 strongly stained 78% of seminomas tested. This heterogeneity in GCTM-2 expression in seminoma is in keeping with recent observations on intermediate filament expression which indicate that seminoma is not a homogeneous entity [16]. Furthermore, heterogeneity in staining for GCTM-2 has been observed within an individual seminoma, as well as between seminoma specimens (M.P.). So far, it has not been possible to separate staining from non-staining seminomas on clinical grounds.

GCTM-2 did not stain a number of non-germ cell tumours that were tested, with the exception of embryonal rhabdomyosarcoma and colorectal carcinoma where moderate staining was observed. Consistent with this was the observation that cultured cell lines from these tumour types expressed the GCTM-2 epitope, as did the choriocarcinoma cell line BeWo. However, the biochemical characteristics and cellular localisation of the antigen in these cell lines are different to that in EC. Enzymatic digestion of carbohydrates prior to immunoblotting of whole cell lysates of GCT 27 revealed that the antigen was sensitive to keratanase digestion. However, immunoblotting of whole cell lysates from HX 18, HX 170 and BeWo visualised protein bands considerably smaller than the 200 kD band in EC, whose electrophoretic mobility was not altered by treatment of the lysate with keratanase. This result suggested that in these cells the antigen was smaller, and lacked the extensive modification with keratan sulphate that was seen in EC. However, the faint immunoreactivity at approximately 200 kD in HX 18 preparations and the enhancement of this staining after keratanase treatment (Fig. 4) suggest the possibility that a proportion of these cells may express the epitope on an antigen similar to the EC proteoglycan. Immunofluorescent staining of live cells with GCTM-2 provided confirmation of the surface localisation of the antigen in EC. By contrast, although all fixed HX 18 cells reacted with GCTM-2, only 30% of live cells did, and no live HX 170 or BeWo cells were reactive, suggesting that in these cell types the antigen is inaccessible to the antibody on the surface, and therefore only internal. It remains to be determined whether the external epitope expressed on 30% of HX 18 cells is carried in the same form as the EC antigen or on the smaller internal antigen.

At one time, it was thought that the strongly hydrophilic proteoglycans had a purely structural or supportive function in tissues. However, most extracellular matrix proteins, and many growth factors can bind to glycosaminoglycans [8], and one core protein has been shown to contain epidermal growth factor like sequences [6]. The importance of proteoglycans in regulating cell growth and differentiation, particularly by binding to growth factors, has only recently come to light [8, 17–20]. The differentiation-dependent expression of the GCTM-2 antigen on human EC cells suggests its possible role in the presentation of cell attachment molecules and growth factors required for stem cell renewal.

The distribution of the GCTM-2 antigen in normal tissues does not conform to any previously described pattern, including that of known keratan sulphate proteoglycans [21]. It was thought for some time that keratan sulphate proteoglycans were confined to cornea and cartilage, but recent evidence suggests that they are more widespread than this [21]. Biochemical analysis of the EC proteoglycan core protein indicates that it is rich in serine, glycine, glutamic acid, and proline, and could be consistent with a number of hexapeptide repeats (S. Cooper *et al.*, Dept Zoology, Oxford University. *Biol J* in press), as found in the large aggregating bovine cartilage proteoglycan [22].

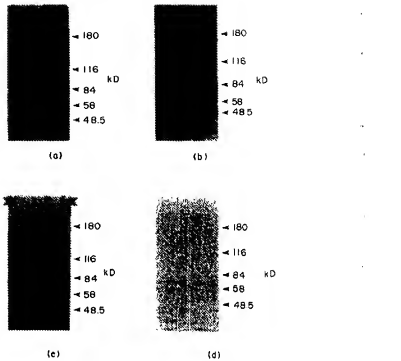

Fig. 4. Immunoblotting with GCTM-2 on whole cell lysates. *Left hand channels*—untreated; *right hand channels*—keratanase treated: (a) GCT 27; (b) HX 170; (c) HX 18; (d) BeWo. Faint immunoreactivity is seen at and above 200 kD to edge of the tracks in (c) (arrows).

However, the aminoacid composition of the core protein of the corneal keratan sulphate proteoglycan distinguishes it from this EC proteoglycan [23]. Furthermore, expression of the EC proteoglycan was not seen in normal fetal cornea and cartilage, and antibodies raised against the keratan sulphate proteoglycans of cornea and cartilage did not show reactivity with human EC cells. (M.P.). The keratan sulphate containing proteoglycan fibromodiulin is distinguished from our EC proteoglycan by its expression in mesenchymal tissues [24]. It is interesting that the expression of keratan sulphate proteoglycans has also been observed following the induction of differentiation of murine F9 EC cells in tissue culture [25].

The GCTM-2 proteoglycan is one of a number of surface antigens found on EC cells, from which it can be distinguished, including SSEA-3, SSEA-4, ABH blood group determinants, TRA-1-60, TRA-1-81, and 8-7D [26–29]. Monoclonal antibody FC 10.2 reacts with an EC membrane glycoprotein of molecular weight 200 kD [30]. The FC 10.2 antigen in EC has been shown to be a carbohydrate chain related to the type 1 blood group sequence [31]. The monoclonal antibody 5T4 was raised against a glycoprotein preparation from human syncytiotrophoblast plasma membrane, and reacts with a 72 kD glycoprotein on syncytiotrophoblast in human hSGCT, but only weakly with EC or yolk sac carcinoma [32]. Finally, a new surface antigen has been identified in EC using the monoclonal antibody 5F9, but it has not been biochemically characterised [33]. That it too is distinct from the GCTM-2 antigen is suggested by the absence of 5F9 staining in seminomas, and by the surface staining in

BeWo cells, though both of these observations require further confirmation.

The differing cellular localisation and biochemical structure of the antigen in EC and in other cells cannot be explained at present. It is possible that the antibody is cross-reacting with an epitope on unrelated proteins in the latter. However, the size of the antigen in these cells is close to that of the core protein in the EC proteoglycan. Furthermore, preliminary results using a second generation monoclonal antibody raised against the purified core protein also indicate reactivity with HX 170 cells on immunofluorescence (M.P.). A more likely possibility is that the GCTM-2 antigen is heavily modified with keratan sulphate and then secreted in EC, but remains internal in other expressing cells, in which the functions of the antigen may be different. In summary, the present results indicate that the GCTM-2 antigen is associated with an extracellular matrix proteoglycan in EC cells and possibly in a proportion of HX 18 cells, but not in other cell types tested. Further studies, using additional probes to the purified proteoglycan, will clarify the nature of the antigen in other tissues.

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